A Novel lux Operon in the Cryptically Bioluminescent Fish Pathogen vibrio salmonicida Is Associated with Virulence


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The cold-water-fish pathogen vibrio salmonicida expresses a functional bacterial luciferase but produces insufficient levels of its aliphatic-aldehyde substrate to be detectably luminous in culture. Our goals were to (i) better explain this cryptic bioluminescence phenotype through molecular characterization of the lux operon and (ii) test whether the bioluminescence gene cluster is associated with virulence. Cloning and sequencing of the V. salmonicida lux operon revealed that homologs of all of the genes required for luminescence are present: luxAB (luciferase) and luxCDE (aliphatic-aldehyde synthesis). The arrangement and sequence of these structural lux genes are conserved compared to those in related species of luminous bacteria. However, V. salmonicida strains have a novel arrangement and number of homologs of the luxR and luxI quorum-sensing regulatory genes. Reverse transcriptase PCR analysis suggests that this novel arrangement of quorum-sensing genes generates antisense transcripts that may be responsible for the reduced production of bioluminescence.

In addition, infection with a strain in which the luxA gene was mutated resulted in a marked delay in mortality among Atlantic salmon relative to infection with the wild-type parent in single-strain challenge experiments. In mixed-strain competition between the luxA mutant and the wild type, the mutant was attenuated up to 50-fold. It remains unclear whether the attenuation results from a direct loss of luciferase or a polar disturbance elsewhere in the lux operon. Nevertheless, these findings document for the first time an association between a mutation in a structural lux gene and virulence, as well as provide a new molecular system to study vibrio pathogenesis in a natural host.

Marine bioluminescent bacteria have been the subjects of considerable interest because of the biochemistry that drives light production and their ability to initiate specific, long-term cooperative symbioses with many species of squids and fishes (20, 35, 45, 51). Less is known about bioluminescence in species of bacteria that have the capacity to produce light yet are found in pathogenic associations with animal hosts (32, 33, 38). It has always been of interest to know whether luminescence plays a role in the biology of such pathogens, either to colonize the hosts or to grow in environmental niches. However, attempts to address such questions were limited because a model system in which to study the relationship between bioluminescence and pathogenesis was not available.

In the five previously characterized species of luminous bacteria (Vibrio fischeri, Vibrio harveyi, Photobacterium leiognathi, Photobacterium phosphoreum, and Photorhabdus luminescens), the six structural genes for bioluminescence are contained within a locus termed the lux operon. With the exception of a duplication of luxB (designated luxF) in one species, these genes are arranged in the order luxCDABEG (1, 9, 16, 28). luxA and luxB, respectively, encode the alpha and beta subunits of luciferase, the enzyme responsible for luminescence. luxC, luxD, and luxE each encode an enzyme required for the synthesis of an aliphatic-aldehyde substrate. luxG is not essential for luminescence but is believed to increase the capacity of the cell to synthesize flavin mononucleotide (FMN) (42). In the luminescence reaction, luciferase converts this aliphatic-aldehyde substrate, oxygen, and reduced FMN (FMNH2) into the corresponding aliphatic acid, water, and FMN, with the concomitant production of light (19, 28). In the absence of the aldehyde substrate, luciferase catalyzes a reaction that yields no light and produces oxygen radicals rather than water (15, 18).

Bacteria that carry the genes for luciferase yet do not produce a detectable level of light in culture have been referred to as cryptically bioluminescent (13), and this phenotype may be quite widespread in the environment (14, 33, 34). Cryptic bioluminescence has been best characterized with the psychrophilic fish pathogen vibrio salmonicida (13), the only bacterium known to cause vibriosis in cold-water, farmed Atlantic salmon (Salmo salar L.), as well as rainbow trout and cod (10, 11).

Cultures of V. salmonicida become visibly luminous only upon the addition of aliphatic aldehyde (an aldehyde group attached to a linear saturated carbon chain) and induce the synthesis of luciferase 10-fold per cell as they approach stationary phase (13). Similarly, when exposed to N-3-oxohexanoyl homoserine lactone, the signal molecule that is in part responsible for quorum sensing and the induction of luminescence in V. fischeri (30, 32), V. salmonicida induces luciferase production 100-fold. Thus, the regulation of the luciferase in V. salmonicida, like that in its close congener V. fischeri...

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plasmid (40) contained in *E. coli* DH5α. *E. coli* and *V. salmonicida* strains were grown to an optical density (OD) at 600 nm of between 0.5 and 0.8 in LB (37°C) and SWT (15°C) broth, respectively. The cells in 1 ml of each culture were pelleted, washed three times in chilled (4°C) SWT, and resuspended in 5 μl of chilled SWT. The resuspended cells were combined, spotted onto a chilled SWT plate, and placed in a 23°C incubator for 6 h. The plate was subsequently incubated at 15°C for another 12 h. The resulting confluent growth of cells was scraped off the plate, resuspended in 1 ml of chilled SWT broth, and incubated with shaking at 150 rpm for 12 h at 15°C. Following the incubation, the suspension was plated onto antibiotic-containing SWT blood agar plates. After 10 days of growth, colonies of *V. salmonicida* transconjugates were streaked for purification.

**Molecular manipulation of the lux region.** (i) Cloning and sequencing of the lux gene cluster. Using standard PCR methods with consensus primers for the luxAB region (forward, 5′-CATGTCATTCGCTA-3′, and reverse, 5′-AGATAAGATCATCA-3′), we generated a PCR product that was cloned into the TA cloning vector (Invitrogen, Carlsbad, CA) to make two plasmid pEN115 (Table 1). A Southern probe analysis based on the internal luxAB sequence in pEN115 was used to screen a library of *Sal* genomic fragments of *V. salmonicida* cloned into the vectors pEVST9 for luxAB-positive clones. One such clone, pEN114, was isolated and sequenced. Because pEN114 lacked the region upstream of luxC, we subcloned the luxAB gene cluster from pEN114 into pEVST9 to make pEN123 and marked luxAB by using the in vitro E EZ:TN-<KAN-2> insertion kit (Epicenter Technologies Inc., Madison, WI) to generate pEN124. pEN124 contained a transposon (Kan') insertion near the middle of the luxAB fragment (886 bp into the luxA open reading frame [ORF]; this transposon has transcriptional terminators at each end. The marked copy of luxAB was introduced into the genome of *V. salmonicida* by triparental mating, and the single recombinant, EN3, was selected by sequentially patching colonies onto SWT-Kan, SWT-Cam, and nonselective SWT blood plates. Genomic DNA from EN3 was purified; digested with ScaI, which cuts upstream of luxC; and ligated into the ScaI site in pEVST9. A Kan-resistant clone derived from this upstream sequence used and used to rescience the *V. salmonicida* library by PCR, resulting in the identification of the plasmid pEN133. The plasmid pEN133 was found to contain a large region immediately upstream of luxC adjacent to the Sall fragment cloned into pEN114. pEN133 was sequenced by standard methods.

(ii) Screening for a double-recombinant luxA mutant. Strain EN3 (Cam and Kan resistant) was grown without Cam selection in SWT. Approximately 10,000 colonies from this culture were screened (Cam sensitive, Kan resistant) for the loss of the integrated pEN124 plasmid by double recombination. Subsequent clones were screened by PCR for the integration of the Kan' marker. The resulting clone, EN4, was examined by sequencing and Southern blot analysis to confirm that a single integration of the Kan resistance marker had occurred in luxA.

(iii) Complementing the luxA mutation. pEN114 was digested with BamHI, and the luxABEG fragment was cloned into the BamHI site of pV08. The

### MATERIALS AND METHODS

**Bacterial strains, media, and culture conditions.** The bacterial strains used in this study are listed in Table 1. *Escherichia coli* DH5α, grown in Luria-Bertani (LB) medium (37°C), was the host for plasmids with CoIE1 or pACYC184 origins of replication. When added to LB medium for the selection of *E. coli* cells carrying a plasmid, ampicillin, chloramphenicol (Cam), and kanamycin (Kan) were used at concentrations of 100, 50, and 50 μg/ml, respectively. *V. salmonicida* strains were originally isolated from diseased Atlantic salmon (*S. salar*), 39. Unless indicated otherwise, the principal strain used in this study was *V. salmonicida* NCMB 2262. *V. salmonicida* cultures were grown at 15°C with shaking at 150 rpm for 2 to 3 days in a complex broth (SWT) that contained 10 g of tryptone, 15 g of agar, and 50 ml of Alsevers sheep blood (Colorado Serum Co., Denver, CO). When added to SWT for the selection of *V. salmonicida* cells carrying a plasmid, Cam and Kan were used at 2 and 150 μg/ml, respectively.

**Triparental mating for *V. salmonicida*.** We developed a version of the triparental mating procedure described by Valla et al. (44), adjusted for the differences in the optimal growth temperatures of *E. coli* and *V. salmonicida* (8). The transfer of plasmids to *V. salmonicida* was performed using pEVST04 as a helper vector.

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<td>pEN135</td>
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**Table 1. Bacterial strains and plasmids**

Cam', chloramphenicol resistance; Erm', erythromycin resistance; Kan', kanamycin resistance.
promoter was isolated (see Fig. 2A). The in vitro EZ::TN plasmid that carried the resulting product, and transformants were selected on LB-Cam medium. A V. fischeri protein (21) and the resulting total RNA fraction (about 1g/H9262 of several bacterial species were identified using DNA Strider V1.2. The Gibbs regulatory genes, respectively (27, 29).

FIG. 1. Organization of bacterial lux genes in V. salmonicida (Vs) and V. fischeri (Vf). VCA represents homologs of the genes for the V. cholerae VCA0181 protein (21) and the V. fischeri VFA0926 protein (36). rbg is a riboflavin synthesis gene (24). Unless indicated otherwise by an arrow, ORFs are predicted to be transcribed from left to right. Black and gray highlighting denotes ORFs corresponding to luciferase genes and lux regulatory genes, respectively (27, 29).

resulting plasmid (pEN134) and the parent vector were separately moved into the luxA mutant EN4 and the wild-type parent by triparental mating. The Gibbs free-energy (ΔG) value for each stem-loop was calculated in units of kilocalories per mole by using the program Mfold (GCC, Madison, WI). ΔG values were calculated for the optimal growth temperatures for the following bacterial species: V. salmonicida (15°C), V. fischeri M1 (30°C), V. harveyi (35°C), and P. leiognathi (35°C).

PCR analysis of the lux region arrangement in five V. salmonicida strains. PCR was used to putatively identify and map the luxR1, luxB, and luxD regions (Fig. 1) of V. salmonicida strains isolated from independent sources (Table 1). PCRs were performed with genomic DNA by using the primer pairs O-EN11 (5'-GC CAGATCAGATTGTTGCTG-3') and O-EN20 (5'-GTCACTTGGCTACCG TCG-3'); O-EN26 (5'-TAAATGATGTTGAGCCAGC-3') and O-EN23 (5'-CTC CATCGCTGCTCAACAG-3'); and O-PEN115M13R1F (5'-GTAATACTAACG GATATGATGTTGAGCCACG-3'). These three primer pairs spanned the regions between luxR1 and luxA, luxA and luxB, and luxB and rbg, respectively.

Genetic complementation of the aldehyde deficiency. A derivative of V. sal monica NA NCMB 2262 was harbored pEN135, which constitutively expressed the V. fischeri ES114 aliphatic-aldehyde synthesis (AAS) genes, luxCDABEG, was constructed. To construct the plasmid pEN135, the V. fischeri lux gene region containing luxRDBEG (luxA was previously deleted) was excised from pKV17 (45) by using Sall and ligated into the V. fischeri cloning vector pVO8 (47). The luxA and luxD genes were removed from the resulting plasmid by KpnI and BglII double digestion, followed by ligation of the digest with a double-stranded oligonucleotide linker that contained KpnI- and BglII-complementing ends; a Sall site was in the middle of the linker. E. coli DH5α was transformed with the resulting product, and transformants were selected on LB-Cam medium. A plasmid that carried the V. fischeri luxDBEG genes under the control of the luxA2 promoter was isolated (see Fig. 2A). The in vitro EZ:TN -KAN-2 insertion kit (Epigenetic Technologies Inc., Madison, WI) was used to create a null insertional mutation located 302 bp into the lux ORF, resulting in pEN135. The pEN135 plasmid was mated into V. salmonicida in a triparental mating as described above.

Measurement of bacterial culture luminescence. Luminescence was measured with a TD-20/20 luminescence meter (Turner Designs Inc., Sunnyvale, CA). The luminescence of late-log-phase cultures were inoculated to an OD of 0.01 and grown to stationary phase. Cultures were inoculated to an OD of 0.01 and grown to stationary phase. The tank was observed twice daily for diseased and dead fish. The identical of the infecting strain (wild type or mutant) was confirmed by sequential plating on SWT-Kan and SWT, and their differential growth was used to calculate the ratio of the mutant cells to the wild-type cells in the infected fish. A similar procedure was used in an in vitro competition experiment performed with SWT broth. Cultures were inoculated to an OD of 0.01 and grown to stationary phase.

Nucleotide sequence accession number. The nucleotide sequence of the V. salmonicida lux gene cluster has been submitted to GenBank (accession no. AF452135).

RESULTS

The structural lux genes are conserved in V. salmonicida. The order of the structural genes of the V. salmonicida lux operon is luxCDABEG, which agrees with the gene orientation in lux operons from other luminescent bacterial species (Fig. 1). The amino acid sequences corresponding to these six genes from V. salmonicida and V. fischeri M1 are also highly conserved, with levels of identity ranging between 65 and 87%
The position where luxI strain NCMB 2262T was found in four other strains of this sequence that do not contain any apparent ORFs (Fig. 2A). MJ1 V. fischeri showed 81% amino acid identity with the product of V. salmonicida luxI structural gene. The product of this luxI gene appears to be transcribed in a direction opposite to that of the structural genes. In addition, there is a novel arrangement in the lux gene cluster. These homologs were designated luxR1 and luxR2, and their predicted protein sequences showed 61 and 63% amino acid identity to the V. fischeri MJ1 luxR gene product, respectively (Fig. 2A). The luxR1 andluxR2 predicted protein sequences showed only 61% amino acid identity to each other, suggesting that there has been considerable divergence since the apparent gene duplication event. Like the luxR gene of V. fischeri, both luxR1 and luxR2 of V. salmonicida appear to be transcribed in a direction opposite to that of the structural lux genes. In addition, there is a luxI homolog adjacent to, but apparently divergent from, the luxR2 homolog (Fig. 2A). The product of this V. salmonicida luxI homolog showed 81% amino acid identity with the product of V. fischeri MJ1 luxI. Between the luxR1 and luxC V. salmonicida ORFs, the position where luxI is located in V. fischeri, are 559 bp of sequence that do not contain any apparent ORFs (Fig. 2A).

The arrangement of the V. salmonicida lux cluster is conserved among different strains. PCR was used to determine whether the lux gene arrangement present in V. salmonicida strain NCMB 2262T was found in four other strains of this species. The three sets of PCRs (see Materials and Methods) covering the luxR1-luxC, luxE-luxR2, and luxR2-ribG regions all produced products of the predicted 1.6-kbp, 1.9-kbp, and 1.9-kbp lengths, respectively, for each of the five V. salmonicida strains (data not shown). These results suggest that the arrangement of the lux gene cluster in the regions amplified is conserved within V. salmonicida.

The aliphatic-aldehyde deficiency can be genetically complemented. One explanation for the aliphatic-aldehyde deficiency and the reduced luminescence of V. salmonicida is that the metabolism of this species is unable to provide the substrate(s) required for the synthesis of aliphatic aldehyde. To examine this hypothesis, V. fischeri AAS genes were expressed in trans in V. salmonicida and the resulting luminescence per cell was determined. V. salmonicida, expressing in trans the AAS gene-carrying plasmid pEN135 (Fig. 3A), was detectably luminous and produced at least 1,200-fold more luminescence than the wild-type strain (Fig. 3B). The addition of exogenous aliphatic aldehyde did not result in a significant increase in luminescence in V. salmonicida cells harboring pEN135 (Fig. 3B). These data suggest that V. salmonicida is not physiologically limited in its ability to produce the substrates required for aliphatic-aldehyde synthesis. In addition, because V. salmonicida cells harboring pEN135 and those harboring the vector plasmid pV08 have the same growth rate (3.3 h per generation), the production of additional aliphatic aldehyde does not appear to be toxic to V. salmonicida. It is unlikely that the collateral presence of a copy of the V. fischeri luxB gene in the construct (Fig. 3A) is responsible for the enhanced luminescence expression observed because aldehyde addition alone increased the luminescence of wild-type cells by several orders of magnitude (Fig. 3B).
The intergenic regions of *V. salmonicida* lux genes share conserved elements with similar regions in *V. fischeri*. The intergenic regions between luxR1 and luxC and between luxR2 and luxI were analyzed for possible transcriptional promoter elements. The putative transcriptional initiation loci upstream of both luxC and luxI in *V. salmonicida* contain substantial similarities to the region upstream of luxI in *V. fischeri* MJ1 (Fig. 4). There are two putative regulatory elements, lux box 1 (between luxR1 and luxC) and lux box 2 (between luxR2 and luxI), in *V. salmonicida*. Both of these elements precede a −10 region that is identical to that found in *V. fischeri* MJ1 (Fig. 4). In addition, *V. salmonicida* lux box 1, *V. salmonicida* lux box 2, and the *V. fischeri* lux box are centered at −42.5, −43.5, and −42.5 bp, respectively, upstream of their predicted transcriptional start sites (6) (Fig. 4). Putative ribosomal binding sites and start codon loci, determined by sequence similarities, are conserved between *V. fischeri* MJ1 and *V. salmonicida* for luxI, luxC, luxA, luxB, and luxG (Fig. 4 and data not shown). However, unlike *V. fischeri* MJ1, *V. salmonicida* has no apparent start codon at the 5′ end of the luxD ORF.

**Stem-loop structures in the *V. salmonicida* lux region are conserved.** To assess how transcription of the *V. salmonicida* lux operon may be terminated, the sequence data were screened for stem-loop structures that may function as rho-independent transcriptional-termination factors. We identified three putative stem-loop structures in the lux gene cluster of *V. salmonicida*. These structures are located in the middle of the luxD and luxA coding regions and at the 3′ end of luxB (Fig. 2A). The sequence of the stem-loop at the luxB terminator in *V. salmonicida* (AAAAGAATGACAGAATTA...)

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**FIG. 3.** Genetic complementation of the aliphatic-aldehyde deficiency in wild-type *V. salmonicida*. (A) The plasmid used for complementation studies, pEN135, contains the *V. fischeri* ES114 AAS genes, luxCDE, as well as the luciferase subunit gene luxB. The other luciferase subunit gene, luxA, is deleted, and luxG (gray) is inactivated by a transposon insertion (triangle). MCS, multiple cloning site. (B) Comparison of luminescence produced by wild-type *V. salmonicida* harboring the vector plasmid pVO8 and that produced by wild-type *V. salmonicida* harboring the AAS gene-carrying plasmid pEN135. Aliquots of cultures (late exponential phase of growth; OD at 600 nm, 0.7 to 0.9) were either immediately measured photometrically or supplemented with aliphatic aldehyde before measurement. The dashed line represents the limit of photometric detection. Data shown are representative of the results of three independent experiments.

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**FIG. 4.** Sequence comparison of the intergenic regions between luxR2 and luxI of *V. salmonicida* (Vs luxR2-luxI), luxR and luxIC of *V. fischeri* MJ1 (Vf luxR-luxIC), and luxR1 and luxC of *V. salmonicida* (Vs luxR1-luxC). The *V. fischeri* luxR-luxIC and *V. salmonicida* luxR1-luxC sequences have been extended into their luxC ORFs. Identical nucleotides in the *V. salmonicida* luxR2-luxI and *V. fischeri* luxR-luxIC sequences are indicated with asterisks above the alignment. Identical nucleotides in the *V. fischeri* luxR-luxIC and *V. salmonicida* luxR1-luxC sequences are indicated with asterisks below the alignment. The highlighted sequences are based on motifs conserved around *V. fischeri* lux genes (17). These regions include a lux box, a −10 promoter region, and known or putative ribosome binding sites (RBS). Start (ATG) and stop (TAG) codons are also highlighted. Gray boxes highlight sequences required for a functional lux box in *V. fischeri* MJ1 (6).
ACTCTGCCATTCTTTTT) is similar to those in other luminous bacteria but was predicted to have greater thermostability ($\Delta G = -15$ kcal/mol) than the equivalent structures in P. leiognathi ($-12$ kcal/mol), V. harveyi ($-10$ kcal/mol), and V. fischeri MJ1 ($-5$ kcal/mol). In V. salmonicida, there are two additional predicted stem-loops, between luxI and ribG (Fig. 2A). It was previously shown by reporter gene analysis that an equivalent stem-loop in V. fischeri, also located between the lux operon and ribG, is a bidirectional transcriptional terminator (42).

**Sense and antisense transcripts of luxE and luxR2 are produced by V. salmonicida.** The organization of the V. salmonicida lux operon, coupled with its aldehyde-deficient luminescence physiology (13), suggested that a long transcript from the rightward luxR2 promoter (Fig. 2A) might exert antisense control over the expression of luxE, one of the genes required for aldehyde synthesis. In support of this hypothesis, when total RNA isolated from growing cells of V. salmonicida was mixed with primers to amplify either sense or antisense luxE or luxR2 transcripts by reverse transcriptase PCR, we detected sense and antisense transcripts of both genes (Fig. 2B; luxR2 data not shown). The relative amounts of the sense and antisense luxE products resulting from the semiquantitative RT-PCR suggested that the level of the sense transcripts was higher than that of the antisense transcripts.

**Complementation of the luxA mutation.** Complementation of the luxA mutation in vitro was observed in the luxA mutant EN4 harboring the plasmid pEN134 compared to EN4 harboring the vector parent plasmid pV08. Specifically, in the presence of decyl aldehyde ($10 \mu l/ml$), EN4 harboring the plasmid pEN134 or pV08 produced $10^9$ or $<10^5$ quanta/s/OD unit, respectively. In the absence of added decanal, an aliphatic aldehyde, EN4 harboring either pEN134 or pV08 produced $5 \times 10^5$ or $<10^4$ quanta/s/OD unit, respectively.

**Atlantic salmon infected with a luxA mutant show delayed mortality.** Dose titration data showed that inoculation with approximately $1 \times 10^6$ CFU of V. salmonicida produced a consistent level of mortality in salmon such that the criterion for a 50% lethal dose would be observed by approximately day 10 (data not shown). This dose is at the upper range of the 50% lethal doses determined previously ($5 \times 10^6$ to $1 \times 10^8$ CFU) by Wiik et al. (49), perhaps because the fry we used were particularly robust. In the large-scale studies, each fish was injected with $1 \times 10^8$ CFU of either the luxA mutant (EN4) or its wild-type parent. Mortality was observed starting at day 2 and continued until day 25 (Fig. 5A). There was no evidence of cross contamination between infected animals: the postmortem examination of fish injected with wild-type cells did not reveal the presence of the mutant strain; the converse was also true. Dead fish did not display pathology on their external surfaces; however, their livers were atypically fatty, and points of hemorrhaging were observed in both the intestines and the kidneys. Levels of mortality in the two branches of the experiment were identical until day 9 (Fig. 5A), at which time the mutant showed a dramatic delay in its ability to kill fish compared to the wild type. The difference between the survival plots for the mutant and the wild type was statistically significant (log rank test, $P = 0.0002$; Wilcoxon test, $P = 0.0015$).

**The luxA mutant is outcompeted by the wild type during infection.** Cells of the luxA mutant EN4 and its wild-type parent were combined in approximately equal numbers (1.07:1), and a portion of this mixed inoculum was injected into the abdominal cavities of 50 fish. The final ratio of mutant cells to wild-type cells in the infection was assessed for each fish that died over the 25-day experiment. Mortality groupings were made according to the duration of infection as a method of identifying stages of the disease (group A, 1 to 5 days; group B, 6 to 10 days; C, 11 to 15 days; and D, 16 to 20 days). The relative competitive index (RCI) of the luxA mutant for each fish was determined by dividing the output ratio for the two strains (mutant cells to wild-type cells) in the head kidney of the dead fish by the input ratio in the mixed inoculum. An RCI of less than 1.0 indicates that the wild type outcompetes the mutant. Each symbol on the graph represents the RCI calculated for one fish, and the geometric mean for each group is indicated by a bar.
tant and wild-type cells (OD, 0.01) were added to SWT broth and replicate cultures were grown overnight. The ratios of mutant to wild-type cells at stationary phase ranged from 0.85 to 0.96.

**DISCUSSION**

We have characterized the luminescence gene cluster of *V. salmonicida* with the goal of understanding the mechanism and role of this organism’s cryptic bioluminescence. To this end, we discovered a unique gene structure that includes an unusual arrangement of quorum-sensing genes. We also showed that a mutation in luxA could attenuate *V. salmonicida* pathogenesis. Although the nature of the association between lux gene expression and virulence remains unknown, the implications of uncovering a new class of virulence factors are significant. The introduction and development of *V. salmonicida* genetics now permits future investigators to explore virulence in what perhaps is the only truly natural vertebrate model system for studying pathogenesis in the genus *Vibrio*.

The prevalence of luciferase genes in Vibrionaceae species that do not produce detectable light has led to the question of what role, if any, there is for the activities encoded by these genes in nonluminescent bacteria. In at least some of these bacteria, the luciferase genes are expressed but the reaction catalyzed by their corresponding proteins is limited by the availability of the aliphatic-aldehyde substrate. The experimental addition of an aliphatic aldehyde results in detectable light emission from these cells (12, 13; unpublished results). Such cryptic luminescence in *V. salmonicida* has been described previously and may occur as a result of several possible explanations: (i) one (or more) of the genes encoding AAS enzymes is absent or nonfunctional; (ii) all the enzymes are synthesized, but the cell produces insufficient substrates for the AAS reaction; and/or (iii) the relative level of expression of the AAS genes is reduced.

The *V. salmonicida* lux operon contains all of the structural lux genes that are required for bioluminescence (Fig. 1). These genes, luxCDAABE, are organized in the typical arrangement observed in other known lux operons. The *V. salmonicida* lux nucleotide sequences are most similar to those of its close relative, *V. fischeri*, and there are no detectable deletions or insertions in the structural lux genes. These data suggest that the aliphatic-aldehyde deficiency is not caused by the absence of the AAS genes.

We also determined that the synthesis of aliphatic aldehyde is not limited by the ability to provide substrates for AAS (Fig. 3B). The precursors for aliphatic aldehyde, namely, saturated long-chain fatty acids and reducing equivalents (5, 43, 48), are apparently readily available for AAS in *V. salmonicida*. Therefore, we explored the hypothesis that reduced or aberrant expression of the AAS genes may explain cryptic bioluminescence in *V. salmonicida*.

The transcription of bioluminescence genes is complex but has been well studied with other models. For example, the 218-bp intergenic region between the start codons for luxR and luxI in *V. fischeri* is the site where the transcription factors LitR (12) and LuxR (41) bind to divergently promote the transcription of luxR and luxI, respectively. Although the exact binding site for LitR is not known, LuxR, in complex with an acylhomoserine lactone autoinducer, binds at a specific region called the lux box (7). The intergenic region between luxRI and luxC in *V. salmonicida* also contains a conserved lux box and a −10 region that is identical to that of *V. fischeri*. In addition, there is a similar translational initiation region for a lux gene (Fig. 4); however, there is no luxI homolog following this locus in *V. salmonicida*. Instead, the region between lux box 1 and luxC in *V. salmonicida* consists of only half the number of nucleotides found between the lux box and luxC in *V. fischeri*. We also detected evidence for a 2-bp frameshift that may result in a premature stop codon (TAG) (Fig. 4). Following this intergenic region in *V. salmonicida*, elements of the translational promoter for luxC are conserved between *V. salmonicida* and *V. fischeri*. Thus, the presence of the conserved transcriptional initiation elements suggests that the transcription of the *V. salmonicida* lux operon may be initiated in a fashion similar to that of the *V. fischeri* operon but that the first gene transcribed in *V. salmonicida* is the luxC homolog and not a luxI homolog. Strong stem-loop structures are predicted in the *V. salmonicida* lux gene cluster that are shared across bioluminescent taxa. The role that these stem-loops play in transcriptional modification remains unknown. Additional findings that *V. salmonicida* produces an as yet undescribed autoinducer (13; data not shown) and encodes a homolog of the *V. fischeri* luminescence regulatory gene litR (12; data not shown) suggest that *V. fischeri* and *V. salmonicida* share other genetic control mechanisms for bioluminescence expression. However, the nature of the expression differs.

Homologs of the luxR and luxI regulatory genes in *V. salmonicida* have a novel arrangement. First, there are luxR homologs located both upstream and downstream of the *V. salmonicida* structural lux genes (Fig. 1). Second, there is a luxI homolog downstream of luxR2. While this arrangement of luxR::luxI is found in *V. fischeri* as well, the location of the regulatory gene pair downstream rather than upstream of the structure genes is unique to *V. salmonicida*. In addition, the bidirectional transcriptional terminator at the end of ribG. This novel genetic structure suggests a transcriptional model in which rightward-sense transcription from luxC to luxE may continue, generating antisense luxR2. Conversely, leftward-sense transcription from luxR2 may produce antisense luxE transcripts. RT-PCR detected both sense and antisense transcripts for luxE and luxR2. These data support the model that antisense gene regulation drives cryptic bioluminescence by reducing the expression of luxE and, therefore, AAS. Preliminary data suggest that a mutation in luxR2 delays the onset of peak bioluminescence but eventually produces a higher peak level than that for the wild type (data not shown). Further mutational and quantitative PCR analyses of each lux gene will be needed to test this model in which antisense RNA contributes to the cryptic bioluminescence phenotype of *V. salmonicida*.

Given the novelty of the arrangement of the luminescence gene cluster in *V. salmonicida*, it is cryptic bioluminescence merely a remnant of an ancestral phenotype, or does it serve a current biological function? All five *V. salmonicida* strains tested appear to share the same lux gene cluster organization. The conservation of the arrangement within the species suggests that a function of the lux gene cluster may exist. To begin
answering questions of functionality, we asked whether an
insertional mutation in luxA would affect the virulence of V. salmonicida.

In a single-strain challenge experiment, the mutagenesis of luxA resulted in a marked delay in mortality among V. salmonicida-infected Atlantic salmon compared to that induced by the wild type. Similarly, in a mixed-strain competition experi-
ment with the mutant and the wild type, the luxA mutant was attenuated 3- to 50-fold depending on the duration of infec-
tion. These data demonstrate that the disruption of luxA attenuates V. salmonicida colonization. However, the mechanism underlying this attenuation is not known. We hypothesize that the attenuation may be directly due to the loss of luciferase, which results in the elimination of the dark luciferase reaction.

The dark luciferase reaction produces toxic oxygen radicals that may serve as a direct virulence agent or a stimulant for pathophysiology. Under this attenuation is not known. We hypothesize that the attenuation may be directly due to the loss of luciferase, which results in the elimination of the dark luciferase reaction. Byers, D., and E. Meighen. 1991. Seasonal variation in presence of Vibrio salmonicida and total bacterial counts in Norwegian fish farm water. Can. J. Microbiol. 35:2815–2818.

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REFERENCES

5. Callahan, S. M., and P. V. Dunlap. 2000. LuxR- and acyl-homoserine-
25. Makemson, J. C. 1986. Luciferase-dependent oxygen consumption by bio-


